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The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3

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Summary. In the present study, a set of three monoclonal antibodies is described, each of which recognizes cells of the monocyte-macrophage lineage in the rat. The tissue distribution, in particular in lymphoid organs, of each of the three monoclonals is determined by immunoenzyme histochemistry on cryostat sections, as well as on cell suspensions. Results show that ED1 recognizes a cytoplasmic antigen in monocytes and in most macrophages, free and fixed. ED2 and ED3 recognize membrane antigens of tissue macrophages, discriminating between distinct subpopulations of macrophages, each with a characteristic localization in the compartments of lymphoid organs. No other cell types except cells of the mononuclear phagocyte system are positive for any of the three monoclonals. Possible relations between the macrophages recognized by this set of monoclonals and dendritic cells are discussed.

INTRODUCTION

The precursors of macrophages originating in the bone marrow differentiate into circulating monocytes which, after leaving the peripheral blood for the tissue compartments, differentiate into macrophages (Van Furth *et al.*, 1972). The macrophages form a heterogeneous population, as expressed by differences in their characteristics, such as morphology, density, enzyme activity, cell surface properties and functional capacities. Together, these characteristics determine the phenotypic appearance of macrophages, which differs with different environmental circumstances, such as the state of activity (Cohn, 1978; Morahan, 1980; Walker, 1976; Beelen, Broekhuis-Fluitsma & Hoefsmit, 1978), the developmental stage of the individual macrophages (Cohn & Benson, 1964), the localization in different tissues (Twomey, Laughter & Brown, 1983; Flotte, Springer & Thorbecke, 1982; Haines *et al.*, 1983) and compartments (Hoefsmit *et al.*, 1980) and often a combination of these factors. Whether these environmental conditions determine the phenotypic appearance of the macrophages (Sorg, 1982) or the origin of the heterogeneity is already determined at bone marrow level (Bursuker & Goldman, 1983) is still a subject of discussion.

Abbreviations: FDC, follicular dendritic cell; IDC, interdigitating cell; i.p., intraperitoneally; PALS, periarteriolar lymphocyte sheath.

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MATERIALS AND METHODS

Animals

Wistar rats and BALB/c mice were obtained for

Centraal Proefdierenbedrijf (T.N.O., Zeist, The Netherlands and kept under routine laboratory conditions.

Preparation of the monoclonal antibodies

BALB/c mice were primed intraperitoneally with rat spleen cells and Freund's complete adjuvant and boosted i.p. after 4 weeks with rat spleen cells and Freund's incomplete adjuvant. Four days after the last immunization, two mice were killed, their spleen cells were pooled and fused with Sp2/0-ag14 myeloma cells according to the method of Köhler & Milstein (1975), modified after Fazekas de St Groth & Scheidegger (1980). The fusion mixture was plated in 96-well culture trays (Costar) with BALB/c peritoneal exudate cells as feeder cells. Hybrids surviving in hypoxanthin, aminopterin and thymidin (HAT) selective RPMI-1640 medium, supplemented with 15% fetal calf serum (FCS, (Hy Clone), Greiner, Alphen, The Netherlands) were tested. Screening of the monoclonal antibodies was performed on cryostat sections of rat spleen by a two-step immunoperoxidase method using peroxidase conjugated rabbit anti-mouse Ig (Dako, Denmark) as second antibody (Dijkstra, 1982). The procedure is described under 'Immuno- and enzyme-histochemistry'. Clones which reacted with putative macrophages in the spleen sections were selected and cloned. Ascites fluid was obtained by intraperitoneal injection of 3×10^6 hybrid cells into BALB/c mice.

Immuno- and enzyme-histochemistry

The following organs were frozen in liquid nitrogen and stored at $-20°$: spleen, thymus, mesenterial lymph node, Peyer's patches, lung (including BALT), liver and bone marrow. Cryostat sections of 8–12 μm were picked up on slides and air-dried. Sections were fixed in acetone for 10 min and air-dried for at least 30 min. Sections were then incubated for 60 min with culture supernatant or with ascites fluid, diluted 1:3000. After washing in 0·01 M phosphate-buffered saline (PBS, pH 7·4), slides were covered with a 1:200 dilution of rabbit anti-mouse Ig peroxidase (Dako) in PBS with 0·2% BSA and 1% normal rat serum for 60 min. After washing in PBS, the slides were stained for peroxidase activity with 3,3′-diaminobenzidine-tetrahydrochloride (DAB, Sigma, St Louis, MO) (0·5 mg/ml Tris-HCl buffer, pH 7·6, containing 0·01% H_2O_2). Control slides were incubated in the same way omitting the first step. Where desired, endogenous peroxidase activity was eliminated by incubation in methanol with 0·03% H_2O_2 (Streefkerk & van der Ploeg, 1974).

Acid phosphatase activity was demonstrated according to Burnstone (Pearse, 1968) with naphthol AS-BI phosphate (Sigma) as substrate and hexazotized pararosaniline as diazonium salt. This procedure was carried out on the cryostat sections following the immunoperoxidase procedure.

Plastic embedded material

Perfusion fixation through the aorta with 0·2% glutaraldehyde and 1% N(3-di-methylaminopropyl)-N′-ethylcarbodiimidhydrochlorid (Merck, Darmstadt, FRG) was performed. Lymphoid organs were removed, cut in pieces of about 0·5 cm thickness, and fixed in the same solution for another 2–3 h at 4°. Vibratome sections of 100 μm thickness were then prepared, washed and incubated subsequently in the antibody (24 hr), conjugate (24 hr) and DAB (1 hr without H_2O_2, 1 hr with H_2O_2) solution as described above for the cryostat sections. After postfixation with OsO_4, the 100 μm sections were dehydrated and embedded in araldite. Selected areas were trimmed out, 1 μm-thick sections prepared on an LKB Ultramicrotome.

Cell suspensions

Lymphoid organs. Spleen, mesenteric lymph node and thymus were cut into small pieces, gently pressed through nylon gauze and suspended in Earl's medium (NPBI, Emmer-Compascuum, The Netherlands).

Peripheral blood. Two to three ml of blood was obtained via cardiac puncture and decoagulated with heparin. Erythrocytes were removed by ammonium shock.

Bone marrow. The femur was excised, the ends cut off, and the marrow eluted and suspended in Earl's medium.

Peritoneal cavity. Peritoneal macrophages were harvested after intraperitoneal (i.p.) injection of 10 ml Earl's medium, which was recollected after 5 min.

These cell suspensions were used to prepare cytocentrifuge preparations; in order to perform glass-adherence, preparations were stained with the monoclonal antibodies, followed by demonstration of acid phosphatase activity. Positive cells were counted and related to the total population of macrophages, as judged by morphology and acid phosphatase activity.

Veiled (dendritic) cells

Cell suspensions of spleen and lymph node were enriched for veiled cells according to Knight *et al.* (1983), using a metrizamide (analytical grade, Nyegaard, Norway) sugar gradient. In cytocentrifuge preparations, cells with characteristics of dendritic cells (irregular shaped, excentric nucleus, extensive cell processes, acid phosphatase activity restricted to a distinct area in the cytocentre) were judged for positivity for either of the three monoclonals.

Determination of isotype

The isotype of the monclonal antibodies was determined in a two-step immunoperoxidase method on cryostat sections of rat spleen using peroxidase conjugates specific for each of the mouse immunoglobulins isotypes (Serotec, Oxford, U.K.) as second step.

RESULTS

ED1, ED2 and ED3 are specific for macrophages

The ED1, ED2 and ED3 monoclonal antibodies all reacted with large, round oval cells or with cells with a dendritic appearance by their slender cell processes. These cells always showed acid phosphatase activity in every organ tested, as demonstrated by combination of the immunoperoxidase procedure with the demonstration of acid phosphatase activity in the same cryostat section. In the splenic red pulp, as well as in the white pulp, cells positive for any of the three monoclonal antibodies contained latex particles 24 hr after intravenous injection (e.g. Fig. 1d). These findings, together with the localization pattern, indicate that these monoclonal antibodies recognize macrophages. They did not recognize other cells, such as granulocytes, lymphocytes, follicular dendritic cells, vascular endothelium, mucosal or bronchial epithelium or liver parenchymal cells.

The distribution and staining pattern of macrophages recognized by each of the three monoclonals is summarized in Table 1. The ED1 monoclonal antibody showed on cryostat sections a granular staining pattern within the cytoplasm of cells. ED2 and ED3 showed a more diffuse staining pattern, not only on the cell bodies but also on the blunt or dendritic cell processes (Fig. 1d, Fig. 2b). This staining pattern is suggestive for membrane staining. The heavy chain class of ED1 is IgG1, and that of ED2 and ED3 is IgG2a. Some relevant findings in different lymphoid organs are described below.

Staining patterns and localization

A. *Spleen (Fig. 1)*

The red pulp macrophages are positive for all three antibodies. ED3, however, stained with red pulp macrophages weakly, the staining intensity increased towards the white pulp (Fig. 1d). The macrophages in the marginal zone and at the inner border of the marginal sinus were strongly positive for ED3, and negative for ED2. ED2 stained a few cells or cell processes in the outer periarteriolar lymphocyte sheath. A patchy staining pattern throughout the white pulp, except in the follicles, was seen with ED3. The distribution in the PALS of ED3-positive cells was similar to that of the Ia-positive interdigitating cells (Dijkstra, 1982).

B. *Lymph node (Fig. 2)*

All medullary macrophages of lymph nodes are positive for ED1 and ED3, only a few are positive for ED2 (Fig. 2). The subsinusoidal macrophages were strongly positive for ED3 (Fig. 2b) and negative for ED2. These branched cells formed a continuous rim at the inner side of the marginal sinus. ED2 stained spindle-shaped cells in the lymph node capsule.

C. *Peyer's patches*

Macrophages in the subepithelial tissue of the intestinal villi stained with ED1 and ED2. ED1 revealed a patchy staining pattern in the interfollicular area (IFA) of the Peyer's patches, ED2 stained branched cells in the IFA and a few cells in the dome area, ED3 stained only small groups of 1–4 cells at the base of the IFA. At the mesothelial side, a rim of spindle-shaped cells stained with ED1 and ED2.

D. *Thymus (Fig. 3)*

The most conspicious staining pattern in the thymus was observed after staining with ED2 (Fig. 3a). In the cortex, large cells were found with cell processes extending between the surrounding lymphocytes (Fig. 3b). These cells showed strong acid phosphatase activity; only very few cells with acid phosphatase activity in the cortex did not stain with ED2. The medulla was absolutely negative for ED2. ED1 showed a patchy staining pattern in cortex and medulla, the highest concentration of patches was found in the corticomedullary region (Fig. 3a). ED3 stained only a few cells very weakly in the thymic medulla.

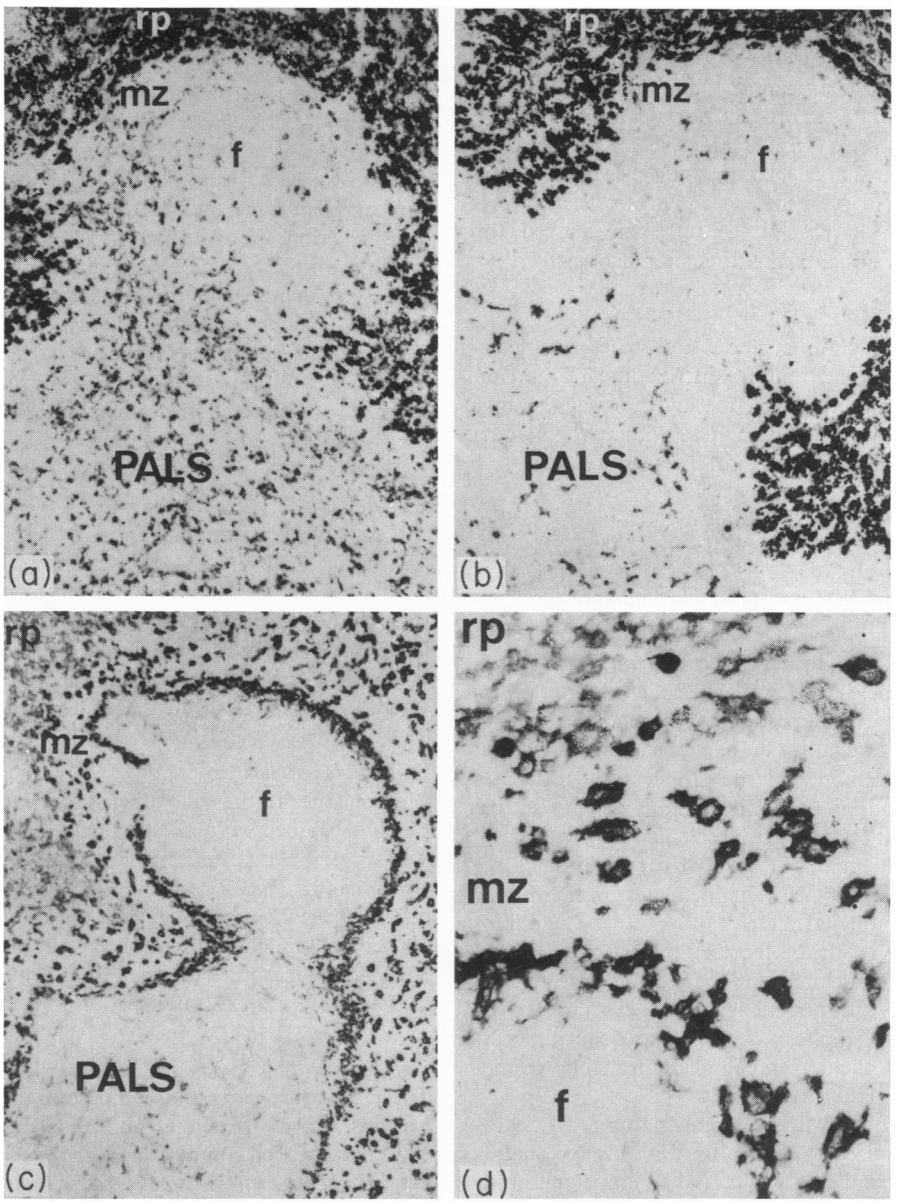

Figure 1. Cryostat sections of rat spleen stained for either of the three monoclonal antibodies in an immunoperoxidase procedure. (a) ED1: a patchy staining pattern is found in the PALS, follicle and marginal zone. The red pulp macrophages show heavy staining for ED1 (magnification ×80). (b) ED2: all red pulp macrophages are stained, as well as a few cells in the outer PALS (magnification ×80). (c) ED3: a rim of strongly positive cells is present at the periphery of the PALS and follicle. Furthermore, large cells with blunt cell processes in the marginal zone are stained. Red pulp macrophages are only weakly positive (magnification ×80). (d) ED3: (in detail). The ED3-positive cells show blunt and dendritic cell processes. The phagocytized latex particles can be easily recognized, especially in the weakly stained cells (magnification ×320). PALS, periarteriolar lymphocyte sheath; f, follicle; mz, marginal zone; rp, red pulp.

Table 1. Distribution and staining pattern of macrophages identified by ED1, ED2 and ED3 in various organs

Monoclonal antibody	ED1	ED2	ED3
Isotype	IgG1	IgG2a	IgG2a
Staining pattern	Granular, patchy cytoplasmic	Diffuse, membrane	Diffuse, membrane
Spleen			
White pulp			
inner PALS	++	−	+Weakly
outer PALS	++	+	+Weakly
follicle	−+	−	−
marg. metallophils	−/+ Weakly	−	+++ Branched
marginal zone	+ Weakly	−	+++ Branched
Red pulp	+++	+++	+++ Weakly
Lymph node			
Cortex			
outer cortex	+Weakly	−	+++ subsinusoidal branched
paracortical area	++	+	−
follicles	−/+	−	−
Medulla	+++	+10–20%	+++
Capsule	+	+	−
Peyer's patches			
Interfollicular area	+++	++	+Small groups 3–4 cells
Dome	+	−	−
Follicle	−	−	−
Villi	+++ Apex	++ Apex basis	−
Lung			
BALT	++	Only at periphery of BALT	−
Perivascular/peribronchial	+	+++	−
Alveolar	+++	−	−
Thymus			
Cortex	++	++ Branched	−
Medulla	++	−	−/+ Weakly
Corticomedullary area	+++	+++	−
Capsule	+++ Branched	+++ Branched	++ Branched
Liver	+++ Branched	+++ Branched	++ Branched
Bone marrow	+++ Monocytes/macrophages	++ Macrophages	−

+++ =(Almost) all acid phosphatase-positive cells stained with the monoclonal antibody.
++ =A considerable number stained.
+ =Few stained.
−/+ =Very few stained, or none at all.

E. *Lung*

In the bronchus-associated lymphoid tissue (BALT), large branched cells were stained by ED1. In the perivascular and peribronchial tissue, as well as at the periphery of the BALT, branched cells, strongly positive for ED2 were found. The alveolar macrophages were strongly positive for ED1 (Fig. 4), and negative for ED2 and ED3.

F. *Liver*

ED1 and ED2 recognize branched cells in the sinuses, probably the cells of Von Kupffer. A smaller number of these cells was recognized by ED3.

G. *Bone marrow*

ED1 stains a considerable number of cells scattered through the bone marrow, comprising large macrophages as well as small monocyte-like cells. ED2 reveals large cells in the bone marrow, often surrounded by erythropoietic cells, together forming a sort of island. ED3 was negative on cryostat sections of bone marrow.

ED1 recognizes a cytoplasmic antigen, ED2 and ED3 recognize a membrane antigen

In plastic sections of lymph node medulla, it was clear

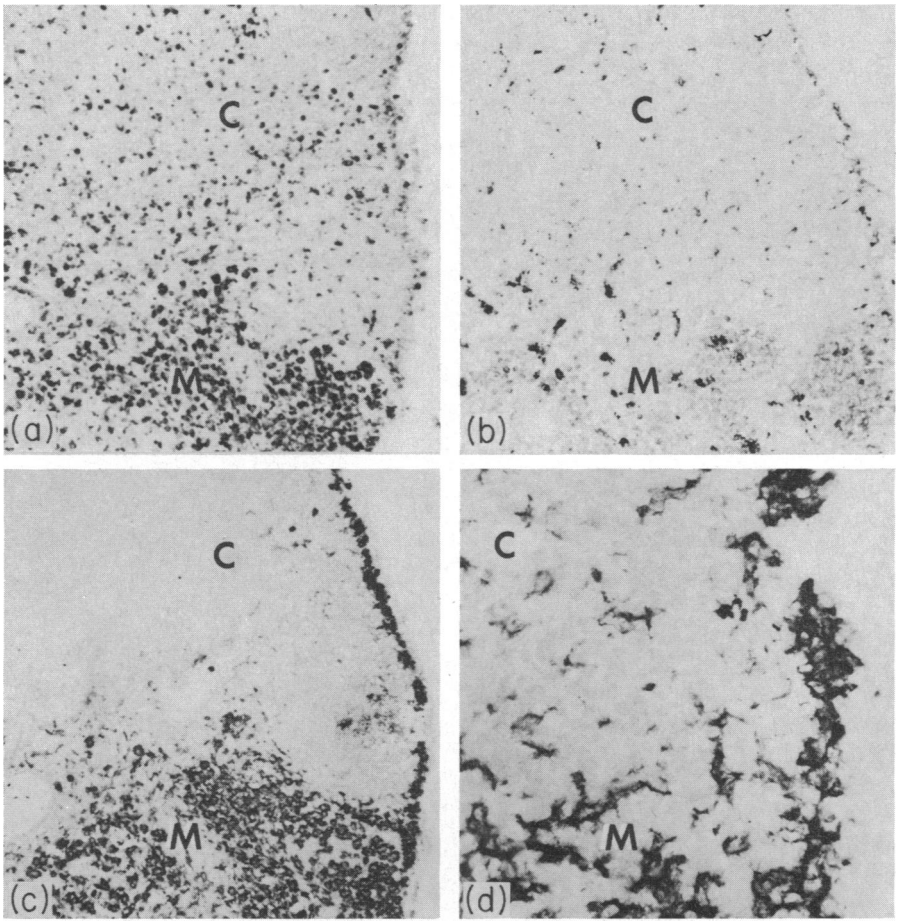

Figure 2. Lymph node. (a) ED1: a patchy staining pattern is found in the cortex; the medullary macrophages show heavy staining for ED1 (magnification × 80). (b) ED2: only very few cells in the lymph node medulla are positive for ED2. Furthermore, some spindle-shaped positive cells are present in the fibrous capsule (magnification × 80). (c) ED3: all medullary macrophages are strongly positive for ED3. Furthermore, a rim of dendritic cells is present at the inner border of the marginal sinus (magnification × 80). (d) ED3: detail of the corticomedullary border, dendritic positive cells are visible at the inner border of the marginal sinus and in the medulla of the lymph node (magnification × 320). C, cortex; M, medulla.

that the antigen recognized by ED1 was located within the cytoplasm of macrophages, whereas ED2 and ED3 showed a membrane localization (Fig. 5).

Cell suspensions

The results on cell suspensions are summarized in Table 2. In bone marrow, monocyte-like cells as well as macrophages were positive for ED1, whereas ED2 stained only macrophages. Monocytes in peripheral blood were positive for ED1 and negative for ED2 and ED3. Peritoneal resident macrophages were strongly positive for ED1, 45% stained only weakly with ED2. In cell suspensions enriched for veiled cells (Knight *et al.*, 1983), almost all cells with characteristics of dendritic cells were stained by ED1, mostly in a distinct area in the cytocentre.

DISCUSSION

In this study, three monoclonal antibodies have been described which recognize different antigenic determinants of rat tissue macrophages. The fact that these

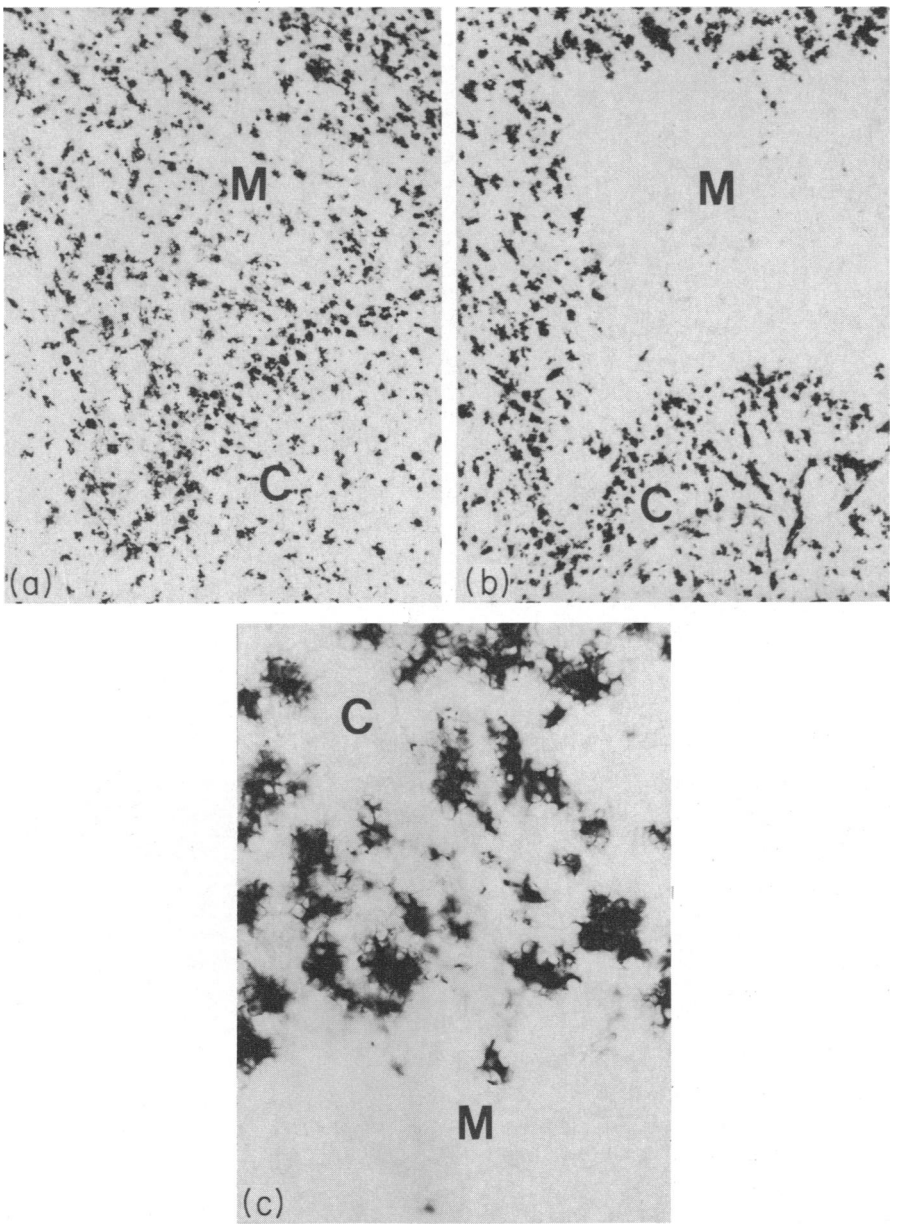

Figure 3. Thymus. (a) ED1: a patchy staining pattern is found in all thymic compartments, the corticomedullary border can be recognized by a relative crowding of positive cells (magnification × 80). (b) ED2: positive dendritic cells are present in the thymic cortex, the medulla does not contain ED2-positive cells (magnification × 80). (c) ED2 (in detail): the ED2-positive cells in the thymic cortex show extensive dendritic cell processes in intimate contact with the surrounding lymphocytes (magnification × 320). C, cortex; M, medulla.

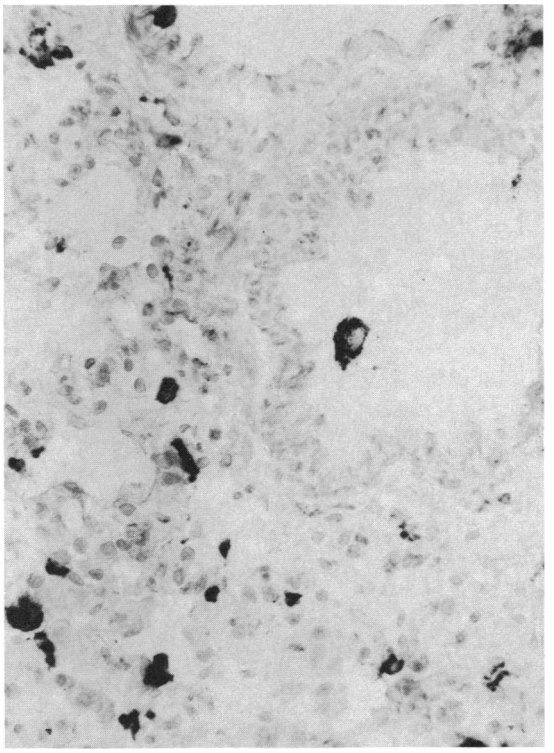

Figure 4. Lung. ED1: free macrophages in bronchiolus and alveoli are strongly positive for ED1 (magnification ×200).

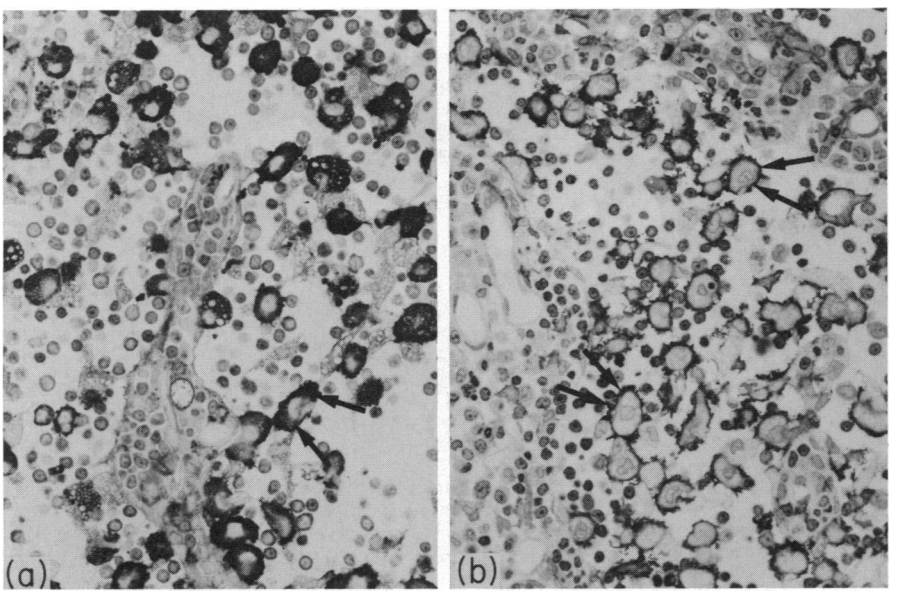

Figure 5. Lymph node medulla, 1 μm plastic section after pre-embedding staining with ED1 (a) and ED3 (b) in an immunoperoxidase method, counterstained with toluidin blue (magnification ×320). (a) ED1 recognizes an intracellularly located antigen (arrow). (b) ED3 shows membrane staining of the medullary macrophages (arrow).

Table 2. Percentages of monocytes/macrophages (as judged by their morphology and acid phosphatase activity) positive for either of the three monoclonal antibodies

	ED1	ED2	ED3	Total MØ*
Spleen	87%	90%	26%	9%
Mesenteric lymph node	80%	25%	50%	5%
Thymus	83%	72%	5%	3%
Peripheral blood	90%	–	–	5%
Bone marrow	82%	72%	–	9%
Peritoneal cavity (resident)	98%	45%	5%	70%
Veiled (dendritic) cells	98%	–	–	–

* Total number of monocytes/macrophages (percentage of nucleated cells) per organ.

antibodies recognize macrophages is assessed by morphological criteria, the tissue distribution and the correlation with acid phosphatase activity. In addition, it has been demonstrated that the spleen cells, positive for any of the three antibodies, are capable of phagocytosis. The monoclonal antibodies did not react with other cell types or tissue structures.

In the compartments of the lymphoid organs, distinct macrophage subpopulations can be recognized exclusively by use of the monoclonal antibodies.

ED1 showed a patchy staining pattern in all compartments of the organs investigated, and recognized most macrophage subpopulations as well as monocytes in the peripheral blood. The majority of both tissue macrophages and free macrophages (alveolar and peritoneal macrophages) are positive for ED1.

ED2 recognizes a membrane antigen of most tissue macrophages, e.g. in connective tissue in all organs tested, the splenic red pulp, the liver (cells of Von Kupffer) as well as a part of the peritoneal macrophages. In the thymus, ED2 stained the cortical macrophages, whereas the medulla was completely negative. The extensive cell processes of the $ED2^+ED3^-$ cells in intimate contact with the surrounding lymphocytes and the exclusive localization in the cortex might indicate a possible role in the maturation of cortical thymocytes, as suggested by Beller & Unanue (1978). Also, in bone marrow, large $ED2^+ED3^-$ cells were found within islands of erythropoietic cells; these central macrophages are thought to have regulatory functions with respect to haemopoiesis (Calderon & Unanue, 1975; Shaklai & Tavassoli, 1979). In peripheral lymphoid organs, only very few cells stained with ED2.

$ED3^+ED2^-$ cells form a particular subpopulation of macrophages, in the splenic white pulp consisting of the marginal zone macrophages (Humphrey & Grennan, 1981) and the marginal metallophils (Snook, 1964), and in the lymph node consisting of the subsinusoidal macrophages and the major portion of the medullary macrophages. This distribution resembles the localization of FITC Ficoll after injection, by which a distinct macrophage subpopulation can be distinguished in mice (Humphrey & Grennan, 1981).

Taken together, these results present evidence that the phenotype of macrophages differs depending on the organ localization. ED1 recognizes a cytoplasmic antigen, distributed more or less randomly in all compartments of the lymphoid organs. ED2 and ED3 recognize membrane antigens, by which separate subpopulations of macrophages can be distinguished in lymphoid compartments. On the other hand, there exists a considerable overlap between the distribution of these two antigens. These results suggest that ED2 and ED3 may recognize differentiation antigens of macrophages.

The tissue distribution of ED1, ED2 or ED3 does not correlate with that of any of the macrophage-antigens described in mice. Neither of the Mac-1, Mac-2 and Mac-3 antigens are located exclusively on macrophages (Flotte *et al.*, 1982). By the localization pattern of Mac-1, Mac-2 and Mac-3, subpopulations of macrophages can also be distinguished (Flotte *et al.*, 1982), but none of these correlates exactly with one of the subpopulations as recognized by ED1, ED2 or ED3. F4/80 antigen is located exclusively on macrophages in mice, with a distribution pattern comparable with ED2 (Hume *et al.*, 1983) but, in contrast with ED2, F4/80 is present in both thymic medulla and cortex. W3/25 monoclonal antibody recognizes macrophages in rat, but T-helper cells are also positive (Barclay, 1981b).

Many of the cells recognized by ED2 and ED3 have a dendritic appearance. This makes it necessary to compare them with the other dendritic cells described in lymphoid organs, i.e. the follicular dendritic cell (Nossal *et al.*, 1968 and the interdigitating cell. None of the antibodies react with the FDC, which is not surprising since considerable evidence exists to suggest that the FDC originates from a reticulum cell (Veerman, 1975; Villena *et al.*, 1983; Dijkstra, Kamperdijk & Döpp, 1984), and not from a member of the mononuclear phagocyte system. There are, however, studies that describe macrophage-related antigens on FDCs (Gerdes *et al.*, 1983).

The interdigitating cells (IDCs) occur in the T-cell areas of lymphoid organs and can be recognized on

ultrastructural level (Veldman, 1979; Veerman, 1974) or by staining for Ia-antigen. Staining with anti-Ia antibody shows a considerable number of strongly positive cells with a dendritic appearance (IDCs) in all T-cell compartments of lymphoid organs (Hoffman-Fezer *et al.*, 1978; Barclay, 1981a; Dijkstra, 1982). In all the T-cell areas investigated, ED1 showed a patchy staining pattern, the distribution of which was similar to that of Ia-positive cells. Both Ia-positive and ED1-positive cells in T-cell areas showed acid phosphatase activity. Dendritic cells (Steinman & Nussenzweig, 1980) or veiled cells (Knight *et al.*, 1983) isolated from lymphoid organs, are thought to be *in vitro* equivalents of IDCs (Dijkstra, 1982). In cell suspensions enriched for veiled cells (Knight *et al.*, 1983), cells with characteristics of dendritic cells are positive for ED1 and negative for ED2 and ED3. Together, these results indicate that interdigitating cells *in vivo* and dendritic cells *in vitro* are both positive for ED1, confirming the suggestion that these cells are closely related and both belong to the mononuclear phagocyte system (Veerman, 1974).

The three monoclonal antibodies described in this study have considerable value in the identification of rat macrophages. In particular, ED2 and ED3 enable us to elucidate the functional aspects and differentiation pathways of the macrophage subpopulations recognized by these unique antibodies.

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